TITLE OF THE INVENTION

Chimeric Filovirus Glycoprotein

This application claims the benefit of an earlier filed application serial no. 60/267,522 filed on January 31, 2001.

INTRODUCTION

Marburg virus (MBGV) was first recognized in 1967, when an outbreak of hemorrhagic fever in humans occurred in Germany and Yugoslavia, after the importation of infected monkeys from Uganda (Martini and Siegert, 1971, Marburg Virus Disease. Berlin: Springer-Verlag; Smith *et al.*, 1982, *Lancet* 1, 816-820). Thirty-one cases of MBGV hemorrhagic fever were identified that resulted in seven deaths. The filamentous morphology of the virus was later recognized to be characteristic, not only of additional MBGV isolates, but also of Ebola virus (EBOV) (Johnson *et al.*, 1977, *J. Virol.* 71, 3031-3038; Smith *et al.*, 1982, *Lancet* 1, 816-820; Pattyn *et al.*, 1977, *Lancet* 1, 573-574). MBGV and EBOV are now known to be distinctly different lineages in the family *Filoviridae*, within the viral order Mononegavirales (Kiley *et al.*, 1982, *Intervirology* 18, 24-32; Feldmann and Klenk, 1996, *Adv. Virus Res.* 47, 1-52).

Few natural outbreaks of MBGV and EBOV disease have been recognized, and all proved self-limiting, with no more than two cycles of human-to-human transmission. However, the actual risks posed by MBGV and EBOV to global health cannot be assessed because factors which restrict the virus to its unidentified ecological niche in eastern Africa, and those that limit its transmissibility, remain unknown (Feldmann and Klenk, 1996, supra). Concern about MBGV is

further heightened by its known stability and infectivity in aerosol form (Belanov *et al.*, 1996, *Vopr. Virusol.* 41, 32-34; Frolov and Gusev Iu, 1996, *Vopr. Virusol.* 41, 275-277). Thus, laboratory research on MBGV and EBOV is necessarily performed at the highest level of biocontainment. To minimize future risk, our primary interest has been the identification of appropriate antigens and vaccine strategies that can provide immunity to MBGV and EBOV.

The filovirus genome is composed of a non-segmented, negative sence, single stranded RNA of approximately 19 Kb. The open reading frames of the genome encode seven viral proteins: GP, the suface viral glycoprotein; NP, the major nucleocapsid protein; VP30, a minor nucleocapsid protein; VP35, a nonstructural protein; VP40 and VP24, internal membrane associated proteins; and L, the viral RNA-dependent RNA polymerase. In addition, Ebola virus encodes and 8th protein termed sGP (secreted glycoprotein). This protein is transcribed from the same open reading frame as full length GP. The function of sGP is unknown.

Vaccination with Marburg or Ebola GP protects from disease and death in rodent models of infection. MBGV GP delivered by Venezuelan equine encephalitis (VEE) virus replicon protected guinea pigs against infection by a homologous strain of Marburg virus (Musoke) (Hevey et al, 1998, Virology 251: 28-37). Similarly, vaccination with EBOV GP delivered by VEE replicon or DNA vaccination also elicited protective immunity in rodent models of Ebola virus infection (Pushko et al., 200, Vaccine 19, 142-153; VanderZanden et al., 1998, Virology 246, 134-144). However, when guinea pigs vaccinated with VEE replicons expressing MBGV (strain Musoke) GP were challenged with a

heterlogous strain of MBGV (Ravn), complete protection was not observed, indicating that genetic variability among filoviruses may present an obstacle in obtaining a broadly cross reactive vaccine. Not surprisingly, vaccination with MBGV GP does not confer protection to challenge with Ebola virus, and vice versa. The GP proteins of MBGV Musoke and MBGV Ravn are 78% identical, compared to only 27% identity between MBGV Musoke and Ebola strain Zaire, indicating that genetic variability among filoviruses may present an obstacle in obtaining a broadly cross reactive vaccine.

Therefore, there is a need for an efficacious boradly cross-reactive vaccine able to protect against multiple filovirus strains.

SUMMARY OF THE INVENTION

The present invention satisfies the need discussed above. The present invention relates to a method and composition for use in inducing an immune response which is protective against infection with different filoviruses. More specifically, the present invention describes a single-component bivalent vaccine protective against both Ebola and Marburg viruses which is cost-effective and efficient to produce, develop and test. The present invention also embodies the production of similar vaccines, whether, bivalent, trivalent, or multivalent able to elicit a protective immune response to multiple filovirus agents in a single-component. Two strains of filoviruses were chosen, Marburg Musoke and Ebola Zaire which are the most divergent of all the filoviruses. If protection against both of these particular most-divergent strains is achieved in a single chimeric GP, chimeras made up of any other

filovirus strains would be expected to also be cross-protective.

In order to elicit an immune response to both Marburg and Ebola, a chimeric glycoprotein was constructed using GP from each of the two viruses. Two forms of the glycoprotein are produced by transcription of the Ebola virus GP gene. Transcription and translation of the genomic copy of the GP gene results in a secreted, truncated form of the membrane-bound GP, termed sGP. The transmembrane GP protein is encoded in two open reading frames (ORFs) via a co-transcriptional editing mechanism whereby the viral polymerase inserts a non-templated adenosine residue at a specific editing site consisting of a run of seven adenosine residues (Volchkov et al, 1995, Virology 214, 421-430; Sanchez et al., 1996, J. Gen. Virol. 73, 347-357). Translation of the resulting mRNA has a frameshift at the editing site, such that sGP is identical to GP up to the editing site (aa295) but divergent thereafter, with a unique 69aa carboxy-terminus. MBGV, in contrast, encodes a single transmembrane GP protein using a single ORF (Will et al., 1993, J. Virol. 76, 1203-1210). The GP proteins of MBGV, (strain Musoke, 681aa, Genbank accession #Z12132 S55429, Feldmann et al., 1992, Virus Res. 24, 1-19) and EBOV (strain Zaire, strain Mayinga, 676aa, Genbank accession # U23187) are 27% identical and 39% homologous. Sequence differences between the two GPs are clustered in a hypervariable region spanning the middle third of the protein, while the N and C termini are more conserved. Both MBGV and EBOV GPs are highly glycosylated and are post-translationally cleaved by a host-cell furin-like protease into two disulfide-linked subunits, termed GP1 and GP2 (Fig. 1) (Volchkov et al., 2000, Virology

286, 1-6; Volchkov et al., 1998, Proc. Natl. Acad. Sci. USA 95, 5762-5767; Becker et al., 1996, Virology 225, 145-155). The transmembrane anchor is located within the GP2 subunit, while the majority of the glycosylation sites are located within GP1 (Feldmann et al., 1999, Arch. Virol. 15, 159-169).

The purpose of our chimeric proteins was to construct a full-length GP (normally consisting of two subunits, GP1 and GP2) containing a subunit from EBOV with the other subunit from MBGV. There was no duplication or deletion of amino acid sequence from the heterologous virus GP in the portions that were cloned together, in order to preserve as much as possible the tertiary structure of the molecule. In other words, the chimeras still have one, and only one, full GP1 subunit and only one full GP2 subunit, as the wild-type EBOV and MBGV GPs do. This was done in order to study the biologic and immunologic properties of the resulting chimera protein in the context of an authentic GP (with respect to 3D or tertiary structure), specifically if any particular function could be attributed to one of the two subunits. This has never been done before for filoviruses,or for any other virus surface glycoprotein. Chimeric GP proteins were constructed by swapping the subunits between EBOV and MBGV. The furin cleavage site of each of these molecules was selected as the junction site for this initial set of chimeras. Several problems were presented by this design.

First, the GP1 and GP2 subunits of wild-type MBGV and EBOV are post-translationally cleaved by a host-cell protease. The cleavage sites for the two proteins are not identical, so putting the cleavage site of one virus in the context of foreign downstream GP2

sequence could potentially disrupt cleavage. Additionally, it would be unknown whether the chimeric subunits, even if cleaved, would still be disulfide bonded as are the wild-type homologous GP1 and GP2 subunits.

Second, the putative structure of GP includes a large, globular, glycosylated GP1 subunit that is most distal to the surface of the membrane, and a smaller GP2 subunit that anchors the GP to the membrane. It is thought that the GP1 subunit is seen by the host immune system, and "shields" the GP2 subunit from being recognized or even being accessible to the host immune system. This is supported by the fact that the highly variable region of GP is located entirely in the GP1 subunit, and regions of variability are thought to correlate with antigenically accessible regions. Therefore one would not expect a chimeric protein with the GP2 subunit from MBGV to protect from MBGV challenge, and conversely one would not expect a chimeric protein with EBOV GP2 to protect from EBOV challenge. One would have predicted that the GP2 subunits are just too small and too hidden to be antigenic.

To begin exploring the possibility of generating a broadly cross-reative vaccine, chimeric EBOV/MBGV GP molecules were constructed in a Venezuelan equine encephalitis (VEE) replicon vector for expression in mammalian cells.

In this study a vaccine delivery system based on a virus replicon was used to identify candidate protective antigens in nonhuman primates. In this vaccine strategy, a gene coding for a protein of interest is cloned in place of the VEE virus structural genes; the result is a self-replicating RNA molecule that encodes its own replicase and

transcriptase functions, and in addition makes abundant quantities of mRNA encoding the foreign protein. When replicon RNA is transfected into eukaryotic cells along with two helper RNAs that express the VEE structural proteins (glycoproteins and nucleocapsid), the replicon RNA is packaged into VEE virus-like particles by the VEE virus structural proteins, which are provided in trans. Since the helper RNAs lack packaging signals neccessary for further propagation, the resulting VEE replicon particles (VRPs) which are produced are infectious for one cycle but are defective thereafter. Upon infection of an individual cell with a VRP, an abortive infection occurs in which the infected cell produces the protein of interest in abundance, is ultimately killed by the infection, but does not produce any viral progeny (Pushko *et al.*, 1997, *Virology* 239, 389-401). The VEE replicon is described in greater detail in U.S. Patent No. 5,792,462 issued to Johnston *et al.* on August 11, 1998.

Results shown here demonstrate that both chimeric GP molecules tested, E/M and M/E, protect guinea pigs against both EBOV and MBGV infection.

Therefore, it is one object of the present invention to provide a chimeric protein comprising GP1 or a portion thereof from one filovirus and GP2 or a portion thereof from another filovirus to produce a chimeric GP protein. By portion, is meant a part of the protein of at least 1 amino acid which is immunologically identifiable with the protein. Different portions of GP1 and GP2 can be used to make up the complete GP chimeric protein. When the chimeric protein is used for a vaccine, the portions of GP1 and GP2 are preferably immunogenic, or elicit an immune response in a subject against the

filoviruses from which these portions were derived. A multivalent vaccine against more than two filoviruses can be designed using immunogenic portions of GP1 and GP2 from more than two filoviruses to produce the chimeric GP protein.

It is another object of the invention to provide a DNA fragment encoding any of the chimeric GP proteins EBOV-GP1 with MBGV-GP2 (E/M), MBGV-GP1 with EBOV-GP2 (M/E), EBOV-GP1 with EBOV-GP2 (E/E), MBGV-GP1 with MBGV-GP2 (M/M), MBGV-RVN GP1 with MBGV-MUS GP2 (Rvn/Mus), MBGV-MUS GP1/MBGV-RVN GP2 (Mus/Rvn) and MBGV-RVN GP1/MBGV-RVN GP2 (Rvn/Rvn) described below.

It is yet another object of the present invention to provide the above DNA fragment as part of a recombinant vector. For example, the vector is a VEE virus replicon vector comprising a VEE virus replicon and a DNA fragments as described above.

It is another object of the present invention to provide a self replicating RNA comprising the VEE virus replicon and any of the EBOV-GP1 with MBGV-GP2 (E/M), MBGV-GP1 with EBOV-GP2 (M/E), EBOV-GP1 with EBOV-GP2 (E/E), MBGV-GP1 with MBGV-GP2 (M/M), MBGV-RVN GP1 with MBGV-MUS GP2 (Rvn/Mus), MBGV-MUS GP1/MBGV-RVN GP2 (Mus/Rvn) and MBGV-RVN GP1/MBGV-RVN GP2 (Rvn/Rvn) described above.

It is another object of the present invention to provide infectious VEE virus replicon particles produced from the VEE virus replicon RNA described above.

It is further an object of the invention to provide an immunological composition for the protection of mammals against filovirus infection such as MBGV and EBOV infection comprising VEE virus replicon particles containing nucleic acids encoding

any portion of the filovirus GP for which protection is desired such as MBGV GP and EBOV GP.

It if yet another object of the present invention to provide an immunological composition for the protection of mammals against filovirus infection comprising VEE virus replicon particles containing nucleic acids encoding a chimeric glycoprotein comprised of any portion of a glycoprotein from any number of filoviruses for which protection against infection is desired. In order to define smaller regions of GP essential for protection, smaller and smaller portions of GP may be swapped between EBOV and MBGV and tested for protection. An understanding of the protective epitopes and GP structure would allow the design of a chimera that contains portions of multiple filovirus GPs resulting in a multivalent vaccine molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Fig. 1 is a schematic of filovirus GP proteins. GP is a highly glycosylated type 1 transmembrane protein. Following cleavage by a furin-like protease, mature GP consists of two disulfide-linked subunits, GP1 and GP2, which are anchored to the virion membrane by the transmembrane domain found within the GP2 subunit. The carboxyl-terminus of GP1 is highly variable among filoviruses.

Fig. 2. MBGV/EBOV Chimeric GP genes. The chimeras were created by cloning the GP1 and GP2 portions by PCR and joined by an engineered unique silent Pvu1 restriction site immediately downstream of the furin cleavage site. MBGV strain Musoke GP protein

is 681 aa (GP1, 436 aa; GP2 245 aa) and EBOV strain Zaire, strain Mayinga GP is 676 aa (PG1 501 aa; GP2 175 aa).

Fig. 3. The VEE replicon system. The VEE structural proteins are replaced with the immunogen of interest (in this case the chimeric GP genes) in a recombinant VEE RNA genome (the replicon), and when the VEE structural proteins are supplied in *trans* the replicon RNA is packaged into defective viral replicon particles (VRP). The packaged replicons are capable of infecting host cells and express large amounts of the foreign immunogen, but are incapable of spreading to new cells.

Fig. 4. Immunoprecipitation of VRP-expressed proteins. Convalescent sera from MBGV infected guinea pigs was used to immunoprecipitate M/M and M/E protein, and convalescent sera from EBOV infected guinea pigs was used to immunoprecipitate E/E and E/M protein from the cell lysate (lanes 1-6) or supernatant (lanes 7-12) of ^{35}S-radiolabeled VRP-infected Vero cells. MBGV GP1 is present in both cell lysate and supernatant from M/M and M/E infected cultures (lanes 3-4 and 9-10), and EBOV GP1 is present in both cell lysate and supernatant from E/E and E/M infected cultures(lanes 5-6 and 11-12). EBOV GP2 is present in the lysate of M/E and E/E infected cells (Lanes 4-5). MBGV GP2 was difficult to detect in lanes 3 or 6; * marks the site of a heterogeneous population of labeled products, the expected size of MBGV-GP2, visible upon overexposure of the gel. Mock infected cell lysate non-specifically bound a protein just under 46 kDa (lane 2), but upon overexposure this discrete band was distinct from that seen in lanes 3 and 6. No proteins nonspecifically bound from mock-infected cell supernatants (lane 8). For reference a

molecular weight marker (mwm) is shown in lanes 1 and 7.

Fig. 5. Guinea Pig Vaccination Schedule. The numbers in the yellow box refer to days pre- and post-virus challenge, set as day 0. Animals were vaccinated twice, 28 days apart, and challenged ~28 days after the second vaccine dose. Animals were anesthetized and bled at the time of each vaccination and before challenge to asses antibody titers. Post-challenge bleeds at days 7 and 14 were used to determine viremia.

Fig. 6. Pre- and Post-MBGV and EBOV Challenge ELISA Titers. Sera from either pre- or post-MBGV (A) or EBOV (B) challenged guinea pigs was assayed for anti-Marburg (MBG) or anti-Ebola (EBO) antibodies using irradiated, sucrose-purified virus as antigen. Dashed line indicates lower limit of assay detection. Numbers above the bars indicate survivors/total; where there were multiple survivors a GMT was calculated. The data indicate that before challenge, anti-MBG titer was detected in the M/M, and M/E groups, while anti-EBO titer was detected in the E/E and E/M groups. Animals that had no detectable antibody titer pre-challenge showed a boost when challenged with homologous virus, indicating these animals were infected.

Fig. 7. Viremias post MBGV (A) and post -EBOV (B) Challenge. Plaque assays were done on serum collected from guinea pigs 7 days post-challenge. The data show that no viremia was detected in animals vaccinated with chimeras containing a GP1 subunit homologous to challenge virus (M/M or M/E challenged with MBGV, E/E or E/M challenged with EBOV). Virus was detectable in the serum of animals vaccinated with chimeras containing the homologous GP2 subunit (E/M

for MBGV, M/E for EBOV), although at much reduced levels compared to animals vaccinated with heterologous VRP-GP (E/E for MBGV, M/M for EBOV). Numbers above each bar indicate viremic/total; where there were multiple viremic animals a GMT was calculated.

Fig. 8. ELISA with detergent-disrupted antigen to detect anti-GP2-reactive antibodies. Reactivity of (A) E/M vaccinated animals with MBG antigen or (B) M/E vaccinated animals with EBO antigen. Antigen was either native (N) or detergent-disrupted (DD; boiled in SDS+2ME) irradiated, sucrose purified virus. The data show that E/M prechallenge vaccinated animals had MBGV-GP2-reactive antibodies detectable only with detergent-disrupted virus, suggesting anti-GP2-specific antibodies which boosted in titer slightly after EBOV challenge. EBO-reactive antibodies were detected at a lower level in M/E vaccinated, pre-EBOV challenge animals. EBO-reactive antibodies were not detected in M/E vaccinated, pre-MBG challenge animals for unknown reasons, however these antibodies were detected (1.7 $\log_{10}$) when Triton X-100 was used to disrupt EBO antigen (data not shown).

Fig. 9. Western blot analysis to detect anti-MBGV-GP2-reactive antibodies. Sucrose purified, irradiated MBGV was electrophoresed under reducing conditions by 4-15% gradient SDS-PAGE. The protein was transferred to nitrocellulose and the blot divided and probed with convalescent guinea pig sera from MBGV or EBOV infected guinea pigs (α-MBGV sera and α-EBOV sera), or sera from pre-challenge animals vaccinated with VRPs expressing M/E (α-M/E) or E/M (α-E/M sera) chimeras. For reference, Biotinylated molecular weight markers were run with each of the four samples. The proteins were detected with Biotinylated goat-anti-

guinea pig secondary antibody and avidin-conjugated Cy3. MBGV-GP2-specific antibodies were clearly present in the sera from E/M vaccinated animals (lane 1).

Fig. 10. Indirect immunofluoresence of Vero cells infected with VRPs expressing either no antigen (mock), M/M (MBGV-GP infected), or E/E (EBOV-GP infected). Antibodies used to detect MBGV or EBOV GP were either convalescent guinea pig sera from MBGV or EBOV infected guinea pigs (α-MBGV sera and α-EBOV sera), or sera from pre-challenge animals vaccinated with VRPs expressing M/E (α-M/E) or E/M (α-E/M sera) chimeras. EBOV-GP2 reactive antibodies were present in the M/E vaccinated animal sera; MBGV-GP2 specific antibodies in the E/M sera reacted at a very low, but detectable, level.

DETAILED DESCRIPTION

In the description that follows, a number of terms used in recombinant DNA, virology and immunology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Filoviruses. The filoviruses (e.g. Marburg virus, MBGV) cause acute hemorrhagic fever characterized by high mortality. Humans can contract filoviruses by infection in endemic regions, by contact with imported primates, and by performing scientific research with the virus. However, there currently are no available vaccines or effective therapeutic treatments for filovirus infection. The virions of filoviruses contain seven proteins which include a surface glycoprotein (GP), a nucleoprotein (NP), an RNA-dependent RNA polymerase (L), and four virion structural proteins (VP24, VP30, VP35, and

VP40). Little is known about the biological functions of these proteins and it is not known which antigens significantly contribute to protection and should therefore be used to induce an immune response by an eventual vaccine candidate. No antigenic cross-reactivity between the Genus Marburg virus and Ebola virus (the only two Genera in the Order Filoviridae). Marburg has species Musoke, Ravn, Popp, and strains Ozolin, Ratayczak, Voege (Musoke and Ravn are the most divergent of the Marburg viruses). Ebola has species Zaire (Strain Mayinga, Strain Zaire, Strain Eckron, Strain Tandala, Strain Kikwit, Strain Gabon) , Sudan (Strain Boniface, Strain Maleo), Reston (Strain Reston, Strain Philippines, Strain Siena, Strain Texas), and Cote d'Ivoire (Strain Cote d'Ivoire). All filoviruses have GP proteins that have similar structure and cleave into GP1 and GP2 subunits. Amino acid homologies of surface glycoproteins of different representatives of the family Filoviridae, made from alignments of different Ebola viruses and one Marburg virus strains are known and can be found in Netesov et al., "Family Filoviridae", In Virus Taxonomy, Seventh Report of the International Committe on Taxonomy of Viruses, Ed.M.H.V. van Regenmortel et al., Academic Press, pp. 539-548.

Replicon. A replicon is equivalent to a full length virus from which all of the viral structural proteins have been deleted. A multiple cloning site can be cloned into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be cloned into this cloning site. Transcription of the RNA from the replicon yields an RNA capable of initiating infection of the cell identical to that seen with the full-length infectious

virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed. This system does not yield any progeny virus particles because there are no viral structural proteins available to package the RNA into particles.

Particles which appear structurally identical to virus particles can be produced by supplying structural proteins for packaging of the replicon RNA in *trans*. This is typically done with two helpers also called defective helper RNAs. One helper consists of a full length infectious clone from which the nonstructural protein genes and the glycoprotein genes are deleted. The helper retains only the terminal nucleotide sequences, the promoter for subgenomic mRNA transcription and the sequences for the viral nucleocapsid protein. The second helper is identical to the first except that the nucleocapsid gene is deleted and only the glycoprotein genes are retained. The helper RNA's are transcribed *in vitro* and co-transfected with replicon RNA. Because the replicon RNA retains the sequences for packaging by the nucleocapsid protein, and because the helpers lack these sequences, only the replicon RNA is packaged by the viral structural proteins and released from the cell. The particles can then by inoculated into animals similar to parent virus. The replicon particles will initiate only a single round of replication because the helpers are absent, they produce no progeny virus particles, and express only the viral nostructural proteins and the product of the heterologous gene cloned in place to the structural proteins.

The VEE virus replicon is a genetically reorganized version of the VEE virus genome in which the structural proteins genes are replaced with a gene

from an immunogen of interest, in this invention, the MBGV virion proteins. The result is a self replicating RNA (replicon) that can be packaged into infectious particles using defective helper RNAs that encode the glycoprotein and capsid proteins of the VEE virus.

Subject. Includes both human, animal, e.g., horse, donkey, pig, guinea pig, mouse, hamster, monkey, chicken, bats, birds and insects such as mosquito.

In one embodiment, the present invention relates to a recombinant DNA molecule that includes a DNA sequence encoding a chimeric protein comprising different regions of filovirus glycoprotein, GP, from different filoviruses. In this application, chimeric proteins were produced using GP from Marburg Musoke (MBGV) and Ebola Zaire Mayanga strain (EBOV) as an example of chimeric proteins from different genera of filoviruses and from the most divergent of all the filoviruses. In addition, chimeric proteins from the most divergent of the Marburg viruses, Musoke (MUS) and Ravn (RVN) were produced as an example of chimeric proteins within the same genus. Accordingly, other species and strains of filovirus can be used to construct a chimeric GP protein since the homology between the filoviruses is known. Percent homologies between the glycoprotein (GP) protein sequences of 5 strains of Marburg virus and Ebola Zaire (Mayinga) are shown below and obtained from Sanchez, A., Trappier, S.G., Stroher, U., Nichol, S.T., Bowen, M. D., and Feldmann, H. (1998). Variation in the glycoprotein and VP35 genes of Marburg virus strains. *Virology* 240:138-146. It is expected that additional filovirus isolates or strains not listed and not yet discovered

can be manipulated as described above and below in order to obtain an chimeric GP protein.

	POP	RYC	MUS	OZO	RAV	EBO-Zaire (Mayinga)
POP						
RYC	100					
MUS	93.5	93.5				
OZO	91	91.0	91.0			
RAV	77.7	77.7	78.1	77.0		
EBO-Zaire (Mayinga)	30.6	30.6	31.1	31.0	30.5	

The complete nucleotide sequence for GP from each of these filoviruses is known: Marburg-Musoke, Feldmann et al., 1992, Virus Res. 24, 1-19 (Genbank accession #Z12132 S55429), Marburg-Ravn, Sanchez et al., 1998, Virology 240, 138-146 (Genbank accession # AF005734), Ebola Zaire Mayinga strain, Sanchez et al., 1993, Virus Res. 29, 215-240 (Genbank accession # U23187).

More specifically, chimeric proteins described in the examples below are presented in Table 1.

Table 1. Chimeric proteins

	SEQ ID NO:	
Chimera	nucleotide	Amino Acid
EBOV-GP1/MBGV-GP2 (E/M)	1	2
MBGV-GP1/EBOV-GP2 (M/E)	3	4
MUS-GP1/RVN-GP2 (M/R)	5	6
RVN-GP1/MUS-GP2 (R/M)	7	8
MBGV-GP1/GP2 (M/M control)	9	10
EBOV-GP1/GP2 (E/E control)	11	12
RVN-GP1/GP2 (R/R control)	13	14

All chimeras contain a restriction site created in the chimeric proteins. The restriction site was

introduced in the three nucleotides flanking either side of the furin cleavage site. The nucleotide changes introduced to engineer the Pvu1 restriction enzyme site are silent in the MBGV GP protein, and change the first amino acid of EBOV-GP2 from glutamic acid to serine (serine is the first amino acid of MBGV GP2).

It is understood that any portion of the a filovirus, e.g. EBOV, GP protein can be fused to any protion of another filovirus's, e.g. MBGV, GP protein as long as the chimeric protein produced induces an effective immune response. Other chimeras from two or more filoviruses could be produced by, for example, swapping portions (say, in 25, 50, 100aa "chunks") of the GP variable region (variable region defined between amino acids ~300-500 of all filovirus GP proteins), or adding the full variable region of one GP to the full sequence from the GP of a second strain of filovirus (tandem variable regions, increasing the overall length of the GP).

For more than two filoviruses, chimeras can be designed to include different amino acid segments from the variable region of multiple filovirus GPs, so the resulting chimeric protein would be expected to protect against all strains represented in the chimeric GP molecule. For example, if a protective epitope can be defined within a 50aa region of protein, then four protective epitopes (ie 50aa chunks) can be combined within the variable region of a single GP to protect against four different filovirus strains and this would apply for either or both GP1 and GP2, e.g. chimeric GP containing the GP2 subunit derived from different filovirus strains, with either a wild type (wt) or chimeric GP1 subunit derived from yet a third filovirus strain (wt) or a

combination of multiple additional filovirus strains (chimera). Any combination of multiple regions of undetermined size along the length of the entire GP protein (including both GP1 and GP2), derived from any combination of any filovirus strains.

The chimeric protein, i.e. the protective antigen, can be cloned into different vaccine vectors for delivery to a subject.

When the DNA sequences described above are in an expression system, such as the VEE replicon described above, the proteins can be expressed *in vivo*. The DNA sequence for any of the MBGV or EBOV virion proteins described above can be cloned into the multiple cloning site of a replicon such that transcription of the RNA from the replicon yields an infectious RNA containing the sequence(s) which encodes the MBGV/EBOV chimeric virion protein or proteins of interest. Use of helper RNA containing sequences necessary for encapsulation of the viral transcript will result in the production of viral particles containing replicon RNA which are able to infect a host and initiate a single round of replication resulting in the expression of the MBGV/EBOV chimeric virion proteins. The sequences encoding the MBGV proteins and the EBOV proteins were cloned into the replicon vector by methods known in the art and described below in Materials and Methods. The VEE constructs (described in Table 2) containing Marburg and Ebola chimeric proteins can be used as a DNA vaccine, or for the production of RNA molecules as described below.

Table 2: Chimeric EBOV/MBGV GP VEE constructs

GP1/GP2	Construct
E/E	EBOV-MAY GP1 (aa1-501)/EBOV-MAY GP2 (aa502-676)
M/M	MBGV-MUS GP1 (aa1-435)/MBGV-MUS GP2 (aa436-681)
M/E:	MBGV-MUS GP1(aa1-435)/EBOV-MAY GP2(aa502-676)
E/M:	EBOV-MAY GP1(aa1-501)/MBGV-MUS GP2(aa436-681)
Rvn/Mus:	MBGV-RVN GP1(aa1-435)/MBGV-MUS GP2(aa436-681)
Mus/Rvn:	MBGV-MUS GP1(aa1-435)/MBGV-RVN GP2(aa436-681)
Rvn/Rvn:	MBGV-RVN GP1(aa1-435)/MBGV-RVN GP2(aa436-681)

Abbreviations:
MGBV=Marburg
EBOV =Ebola
MUS = Marburg strain Musoke
RVN = Marburg strain Ravn
MAY =Ebola strain Zaire-Mayinga
GP1 =GP1 subunit of GP protein
GP2 =GP2 subunit of GP protein
aa =amino acid (in these instances referring to the amino acid number/ position in the wild-type sequence)

In another embodiment, the present invention relates to RNA molecules resulting from the transcription of the constructs described above. The RNA molecules can be prepared by *in vitro* transcription using methods known in the art and described in the Examples below. Alternatively, the RNA molecules can be produced by transcription of the constructs *in vivo*, and isolating the RNA. These and other methods for obtaining RNA transcripts of the constructs are known in the art. Please see Current Protocols in Molecular Biology. Frederick M. Ausubel et al. (eds.), John Wiley and Sons, Inc. The RNA molecules can be used, for example, as a direct RNA vaccine, or to transfect cells along with RNA from helper plasmids, one of which expresses VEE glycoproteins and the other VEE capsid proteins, as

described above, in order to obtain replicon particles.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to mouse and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of MBGV or EBOV virion proteins, such as glutathione S-transferase. The recombinant molecule can be suitable for transfecting eukaryotic cells, for example, mammalian cells and yeast cells in

culture systems. *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as CHO cells, vero cells, and COS cells to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus (CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art.

The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein cloned into the VEE replicon, or a source of RNA transcribed from the replicon as described above, or a source of replicon particles.

In a further embodiment, the present invention relates to a method of producing the recombinant or chimeric protein which includes culturing the above-described host cells, under conditions such that the DNA fragment is expressed and the recombinant or fusion protein is produced thereby. The recombinant or chimeric protein can then be isolated using methodology well known in the art. The recombinant or

chimeric protein can be used as a vaccine for immunity against infection with MBGV or EBOV or as a diagnostic tool for detection of MBGV or EBOV infection. The transformed host cells can be used to analyze the effectiveness of drugs and agents which inhibit MBGV or EBOV virus function, such as host proteins or chemically derived agents or other proteins which may interact with the virus to inhibit its replication or survival.

In another embodiment, the present invention relates to a single-component vaccine protective against several filoviruses. In a specific embodiment the filoviruses are MBGV and EBOV. It is understood, that protection against infection with other filoviruses can be achieved by using the concept of the present invention described for MBGV and EBOV since all filoviruses have GP proteins that have similar structure and cleave into GP1 and GP2 subunits. Other filoviruses include Genus Marburg virus and Ebola virus (the only two Genera in the Order Filoviridae). Marburg has species Musoke, Ravn, Ozolin, Popp, Ratayczak, Voege. Ebola has Strain Zaire, Sudan, Reston, and Cote d'Ivoire (with 1-6 species under each strain). A specific vaccine of the present invention comprises one or more replicon particles derived from one or more replicons encoding one or more MBGV and EBOV glycoproteins.

The present invention also relates to a method for providing immunity against MBGV and EBOV virus said method comprising administering one or more replicon particles containing any combination of the MBGV and EBOV glycoproteins to a subject such that a protective immune reaction is generated. Even though the MBGV strain Musoke was used in the examples below, it is expected that protection would be afforded using

virion proteins from other MBGV strains and isolates, and/or other EBOV strains and isolates.

Vaccine formulations of the present invention comprise an immunogenic amount of a replicon particle, resulting from one of the replicon constructs described above, or a combination of replicon particles as a multivalent vaccine, in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the replicon particles sufficient to evoke an immune response in the subject to which the vaccine is administered. An amount of from about 10^5 to 10^8 or more replicon particles per dose with one to three doses one month apart is suitable, depending upon the age and species of the subject being treated. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

Administration of the replicon particles disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by in ovo injection in birds, orally and by topical application of the virus (typically carried in the pharmaceutical formulation) to an airway surface. Topical application of the virus to an airway surface can be carried out by intranasal administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally). Topical application of the virus to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the replicon as an aerosol suspension, and then

causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed.

When the replicon RNA or DNA is used as a vaccine, the replicon RNA or DNA can be administered directly using techniques such as delivery on gold beads (gene gun), delivery by liposomes, or direct injection, among other methods known to people in the art. Any one or more constructs or replicating RNA described above can be use in any combination effective to illicit an immunogenic response in a subject. Generally, the nucleic acid vaccine administered may be in an amount of about 1-5 ug of nucleic acid per dose and will depend on the subject to be treated, capacity of the subject's immune system to develop the desired immune response, and the degree of protection desired. Precise amounts of the vaccine to be administered may depend on the judgement of the practitioner and may be peculiar to each subject and antigen.

The vaccine may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses

expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

The following MATERIALS AND METHODS were used in the examples that follow.

Cells and viruses. BHK, Vero, and Vero E6 cells were maintained in minimal essential medium with Earle's salts (EMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 2% penicillin (20,000IU/ml)/streptomycin (20mg/ml). MBGV strain Musoke was isolated from a human case of infection in Kenya in 1980 (Smith et. al., 1982, Lancet 1, 816-820). This virus was adapted to guinea pig lethality by eight passages in strain-13 guinea pigs followed by three passages and plaque purification on Vero E6 cells (Hevey et. al., 1997, Virology 251, 28-37). EBOV strain Zaire strain Mayinga was passed six times in VeroE6, cells, twice on Vero cells, and an additional time on Vero E6 (Jharling et al., 1996, Arch. Virol. Suppl. 11, 135-147). MBGV and EBOV were grown in Vero E6 cells cultured to confluency in roller bottles. Cells were infected with MBGV or EBOV at a low moi (<0.05) and virus allowed to grow for 7 days, with EMEM+10%FBS replaced at 4 days post infection. Cell supernatant was harvested and clarified by centrifugation for 15 min at 1500*g*, and virus yield was determined by plaque assay. To concentrate virus for use as ELISA antigen, virus was pelleted by additional centrifugation of the culture supernatant at 10,000*g* for 30 min. Pelleted virus was resuspended in TNE (10mM Tris pH 7.4, 150 mM NaCl, 1mM EDTA), layered over 20-60% sucrose-TNE gradient, and centrifuged at 38,000 rpm (SW41 rotor) for 4 hr. The visible virus band was collected, aliquoted, and irradiated (6MR, ^{60}Co source). Virus was safety tested for the absence of infectivity in tissue culture.

Cloning of chimeric GP genes. Chimeric GP proteins were constructed by swapping the GP1 and GP2 subunits between EBOV strain Zaire Mayinga strain and MBGV strain Musoke. The GP1 and GP2 portions were cloned by PCR and joined by introduction of a unique Pvu1 restriction site created in the three nucleotides flanking either side of the furin cleavage site. The nucleotide changes introduced to engineer the Pvu1 restriction enzyme site are silent in the MBGV GP protein, and change the first amino acid of EBOV-GP2 from Glu to Ser (Ser is the first aa of MBGV GP2). The GP1 forward and GP2 reverse primers were tailed with HindIII and EcoRI restriction enzyme sites, respectively, and following ligation of the GP1 and GP2 PCR products at the Pvu1 restriction site, the full-length GP genes were ligated into the VEE shuttle vector using HindIII and EcoRI. The chimeric and wt GPs were then excised from shuttle vector and ligated into the VEE replicon plasmid using ClaI, and screened for orientation before expression. The VEE constructs are described in Table 2.

The primers used for cloning were as follows: GP1 forward (containing HindIII) EBOV (5'-CAA GCT TCA ATG GGC GTT ACA-3', SEQ ID NO:15), MBGV (5'-AAG CTT AAC ATG AAG ACC ACA T-3', SEQ ID NO:16); GP2 forward (containing Pvu1) EBOV (5'-GAC GAT CGG CAA TTG TCA ATG-3', SEQ ID NO:17), MBGV (5'-AGC GAT CGA TCC TCT GGA G -3', SEQ ID NO:18); GP1 reverse (containing PvuI) EBOV (5'-GCC GAT CGT CGA GTT CTT CT-3', SEQ ID NO:19), MBGV (5'-GAT CGA TCG CTT TCT TCT G-3', SEQ ID NO:20); GP2 reverse (containing EcoRI) EBOV (5'-TGA ATT CAA CTA AAA GAC AAA TTT G-3', SEQ ID NO:21), MBGV (5'-CGA ATT CCG TTA TCC GAT ATA T-3', SEQ ID NO:22). The original EBOV GP gene was provided by Dr. Tony

Sanchez, CDC, Atlanta, GA; MBGV GP gene was provided by Drs. A. Sanchez and H. Feldmann.

The primers used for cloning the following three chimeras MBGV MUS-GP1 with MBGV RVN-GP2 (M/R), and MBGV-RVN-GP1 with MBGV MUS-GP2 (R/M) and MBGV RVN-GP1 with MBGV RVN-GP2 (R/R) were as follows: GP1 forward (containing HindIII) MBGV MUS (5'-AAG CTT AAC ATG AAG ACC ACA T-3', SEQ ID NO:23) MBGV RVN (5-AAG CTT CGA CAT GAA GAC CAT AT-3', SEQ ID NO:24); GP2 forward (containing Pvu1) MBGV MUS (5'-AGC GAT CGA TCC TCT GGA G -3', SEQ ID NO:25), MBGV RVN (5'-AAC GAT CGA TTT TCT GGA A-3', SEQ ID NO:26); GP1 reverse (containing PvuI) MBGV MUS (5'-GAT CGA TCG CTT TCT TCT G-3', SEQ ID NO:27), MBGV RVN (5'-AAA TCG ATC GTT TCT TTC TAA AG-3', SEQ ID NO:28); GP2 reverse (containing EcoRI) MBGV MUS (5'-CGA ATT CCG TTA TCC GAT ATA T-3', SEQ ID NO:29), MBGV RVN (5'-CGA ATT CTG TCA TCC AAT GTA T-3', SEQ ID NO:30). M/R, R/M, and R/R were cloned into shuttle vector with HindIII EcoRI, then moved to replicon vector with ClaI.

VEE replicons: Production and Quantification. Viral replicon particles (VRPs) were packaged as described previously (Pushko et al., 1997, Virology 239, 389-401). Briefly, replicon RNA was transcribed in vitro which contains the VEE non-structural protein genes and the wt or chimeric GP gene. The replicon RNA was cotransfected, along with two *in vitro* transcribed helper RNAs coding for the VEE glycoproteins and the VEE capsid protein, to provide the structural proteins in *trans*, into BHK cells. The replicon RNA contains the authentic VEE packaging signal (which the helper RNAs lack) and is therefore incorporated into VRPs. Infectious, replication-incompetent replicons were collected from the culture supernatant 24 and 40 hrs after transfection, and concentrated by pelleting

through a 20% sucrose-TNE cushion (10mM Tris pH7.2, 150mM NaCl, 1mM EDTA) for 4 hr at 25,000 rpm (SW28 rotor), and resuspended in 1ml PBS. Packaged replicons were titered by serial dilution on Vero cells cultured in chamber slides (LabTek, Nunc, Inc., $1x10^5$ cells/well). VRPs were allowed to infect the Vero cells for 18 h at 37°C to allow the wt and chimeric GPs to be expressed. The cells were then fixed for 20 min in acetone, and antigen-positive cells detected by indirect immunofluorescence (IFA) as described previously (Schmaljohn et al., 1995, Virology 206, 963-972) using polyclonal guinea pig anti-EBOV and guinea pig anti-MBGV. The negative control replicon expressing Lassa NP protein was quantitated similarly, as described previously (Hevey et al., 1998, Vaccine 20, 586-593).

Immunoprecipitation. VRP-expressed proteins were assayed by immunoprecipitation using polyclonal guinea pig anti-EBOV (kindly provided by Joan Geisbert, USAMRIID, and from E/E vaccinated, EBOV challenged convalescent animals presented here) or guinea pig anti-MBGV (from M/M vaccinated, MBGV challenged convalescent animals presented here and Hevey et al., 1997, supra). To generate radiolabeled wt or chimeris GP protein, Vero cells were infected with VRP at moi=3 in 6-well plates and incubated at 37°C for 18hr. Complete medium was removed, cells were starved for 30 min in 1X MEM cystine/methionine free media (ICN, Inc., Aurora, OH), and then labeled for 4 hr in cys/met-free media supplemented with 50 uCi/ml S^{35}-cystine, 50uCi/ml S^{35}-methionine, and 2% FBS. Supernatant was collected, cells were washed once with Zwittergent wash buffer (0.4% Zwittergent, 0.5M NaCl, 10mM Tris pH 8.0, 1mM EDTA) and lysed by incubation in 500 ul Zwittergent lysis buffer (4% Zwittergent, 0.5M

NaCl, 10mM Tris pH 8.0, 1mM EDTA) for 5 min on ice with rocking. To prepare antibody, polysclonal sera was adsorbed to magnetic Protein A beads (Dynal, Oslo, Norway) in a ratio of 15ul antibody:50ul bead slurry per sample, for a minimum of1 h at 4°C with continuous rocking. Unbound antibody was washed from beads with 0.5 ml Zwittergent wash buffer using a magnetic particle concentrator (MPC; Dynal) for separation of magnetic beads form supernatant. Beads were resuspended to a volume of 50ul/sample in Zwittergent lysis buffer (0.4% Zwittergent, 0.5M NaCl, 10mM Tris pH 8.0, 1mM EDTA) and 50ul beads then added to 50 ul cell extract or cell supernatant. Antigen was allowed to bind the antibody-coated magnetic beads for 4 h at 4°C with continuous rocking. Beads were washed three times in Zwittergent wash buffer and then resuspended in 30 ul 2X Laemmli sample buffer (BioRad, Hercules, CA) + 5% 2-mercaptoethanol. Samples were then incubated for 5 min at 95°C, the beads magnetically separated, and 15 ul supernatant was electrophoresed on a 5-15% gradient SDS-PAGE.

Guinea Pig Vaccination. To test protective efficacy, five groups of inbred strain 13 guinea pigs, consisting of 12 animals per goup, received 2 doses of 10^6 focus forming units (ffu) of replicon expressing M/M, M/E, E/E, or E/M 28 days apart (a schedule that has been previously demonstrated to be protective; Hevey et al., 1998) (Fig. 5). Animals were vaccinated subcutaneously with 0.5ml total volume administered at two dorsal sites. As a control, animals were immunized with Lassa NP, an irrelevant VRP-expressed antigen. Animals were anesthetized and bled at the time of each vaccination (days 0 and 28) and before challenge (~day 50) in order to measure antibody responses of vaccinated animals by ELISA. Half the immunized

animals in each group were challenged with a guinea pig adapted Marburg virus (strain Musoke) and the other half with a guinea pig adapted Ebola virus (strain Zaire-Mayinga) 23-28 days after the second vaccine administration. Heparinized plasma was collected from the retroorbital sinus of anesthetized animals on days 7 and 14 post-challenge in order to measure serum viremia. Surviving animals were monitored for 30-45 days post-challenge, anesthetized, and exsanguinated to obtain convalescent serum.

ELISA. Antibody titers against EBOV and MBGV GP were determined pre-and post-challenge by indirect ELISA. Antigen consisting of sucrose-purified, irradiated MBGV or EBOV was used to coat PVC 96-well microtiter ELISA plates (Dynex Technologies Laboratories, Chantilly, VA) at a dilution empirically determined to result in optimal reactivity, usually 1:1000-1:2000, 50 ul/well in PBS incubated overnight at 4°C. Nonspecific binding was blocked by incubation with 200 ul/well PBS+ 0.02% Tween-20 (PBST) with 5% nonfat dry milk, for 1 hr at room temperature. Plates were then washed five times with 200 ul/well PBST. Serum was tested by making half-log dilutions (3.16-fold) in PBST+5% milk and 1% FBS, 50ul/well, aliquoted in duplicate down the plate, generating eight concentrations for each sample. Primary antibody was incubated with the antigen for 1 hr at room temperature, followed by washing five times in PBST. Secondary antibody was 100 ul/well of horseradish peroxidase (HPO)-conjugated goat anti-guinea pig IgG(H+L) (Kirkegaard and Perry laboratories, Gaithersburg, MD), used at a1:2000 dilution in PBST+5% milk and 1% FBS, incubated for 1 hr at room temperature, followed by washing five times in PBST. Colormetric peroxidase substrate, 2,2'-azinobis-[3-

ethylbenzothizoline-6-sulfonic acid] diammonium salt (ABTS; Kirkegaard and Perry laboratories, Gaithersburg, MD), was added, 100ul/well. After 30 min at room temperature the optical density at 405nm of each well was determined using SpectroMAX340 (Molecular devices, Sunnyvale, CA). Endpoints were calculated for each dilution series using SOFTmaxPro software (Molecular devices Corp.), using a curve-fit of background-subtracted mean OD versus dilution, and subsequent extrapolation of the dilution at which OD=0.2. Results are shown in Figs 4-6 are from a single assay each to allow valid comparisons between groups.

Plaque Assay. Plaque assays were performed as described previously (Moe et al., 1981, J. Clin. Microbiol. 13, 791-793). Briefly, ten-fold serial dilution of samples to be tested were made on Vero E6 cells cultured in 6-well plates. One hundred microliters of each virus dilution was adsorbed to monolayers for 1 hr at 37°C, followed by a 0.5% agarose-1X EBME overlay. Six days post infection a second overlay of 20% Neutral Red solution in 0.5% agarose-1X EBME was added to visualize plaques. The neutral red overlay was incubated for an additional 24 hr at 37°C, and plaques were counted on day 7.

Western blot. Sucrose purified, irradiated EBOV or MBGV diluted 1:50 in PBS was heated for 5 min at 95°C in 2X Laemmli sample buffer (BioRad, Hercules, CA) + 5% 2-mercaptoethanol prior to separation by gel electrophoresis. Proteins were electroblotted onto Immobilon-P nitrocellulose (Millipore, Bedford, MA). After blocking in PBST+5% nonfat dry milk, the blots were washed and incubated with vaccinated, pre-challenge guinea pig sera in PBS+0.02% Tween-20 (PBST) for 1 hr with rocking. Secondary antibody was a

1:1000 dilution of biotinylated goat-anti-guinea pig IgG(H+L) (Kirkegaard and Perry laboratories, Gaithersburg, MD), incubated for 1 hr in PBST with rocking. Protein was detected with a 1:1000 dilution of avidin-conjugated Cy3 (Kirkegaard and Perry laboratories, Gaithersburg, MD), and visualized using the Typhoon 8600 Scanner (Molecular Dynamics, Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Indirect immunofluorescence assay (IFA). Cultured Vero cells were infected with replicons expressing the wt or chimeric GP proteins, or the Lassa NP protein. One day post infection the cells were trypsinized, washed with PBS, dried on 10-well teflon-etched spot slides (Cell-Line Associates, Inc., Newfield, NJ), and fixed in acetone for 20 min at -20°C. Antigen was detected by incubation with polyclonal guinea pig sera for 30 min at room temperature, diluted 1:50 in PBS + 1%FBS for a total of 20ul/spot. Slides were then washed for 30 min submerged in PBS with stirring. A secondary FITC-conjugated anti-guinea pig antibody (made in-house at USAMRIID) was used at a 1:40 dilution in PBS+1%FBS, 20ul/well, incubated for 30 min at RT, and the slides washed again for 30 min in PBS. Fluorescing cells were imaged using Carl Zeiss Imaging system (AxioPlan light microscope, AxioCam CCD camera, and AxioVision 2.05 image capture software, 1999; Carl Zeiss, Inc., West Germany).

Example 1

Chimeric GP VEE replicon constructs

Filovirus GP proteins are post-translationally cleaved by a host cell furin-like protease into two disulfide-linked subunits, GP1 and GP2 . Chimeric GP proteins were constructed by swapping the GP1 and GP2 subunits between EBOV strain Zaire (Mayinga) and MBGV

strain Musoke. The furin cleavage site of each of these molecules was selected as the junction site as an attempt to minimize disruption of protein folding and tertiary structure and increase the likelihood of forming a stable molecule. Two chimeras were made: EBOV-GP1 with MBGV MUS-GP2 (E/M), and MBGV MUS-GP1 with EBOV-GP2 (M/E). As controls, the GP1 and GP2 portions of EBOV and MBGV were also cloned with the same Pvu1 restriction site, creating the equivalent of wild-type (wt) molecules EBOV-GP1 with EBOV-GP2 (E/E), and MBGV MUS-GP1 with MBGV MUS-GP2 (M/M) (Fig. 2).

The chimeric molecules were cloned into the VEE replicon vector which offers the advantage of high protein expression levels in mammalian cells as well as serving as a proven vaccine vector (Hevey et al., 1998, supra; Pushko et al., 1997, supra). As depicted in Figure 3, the VEE replicon vector system replaces the VEE structural proteins with the immunogen of interest (in this case the chimeric GP molecules) in a recombinant VEE RNA genome (the replicon RNA). The packaged viral replicon particles (VRPs) are capable of infecting host cells and express large amounts of the foreign immunogen, but are incapable of spreading to new cells.

Replicon-driven expression of the EBOV/MBGV wt and chimeric GP proteins was confirmed by immunoprecipitation using polyclonal guinea-pig anti-EBOV and guinea pig anti-MBGV sera (Fig.4). Protein products of the expected sizes were specifically precipitated by the appropriate antisera, indicating antigenicity of the proteins was maintained. The apparent size of filovirus glycoproteins, approximately 170 kDa, is much larger than the predicted molecular weight because of the abundant glycosylation, and a heterogenous population of post-

translationally modified GPs is typically observed by electrophoresis (Feldmann et al., 1991, Arch. Virol. 15, 159-169; Feldmann et al., 1994 Virology 199, 469-473; Volchkov et al., 1998, Proc. Natl. Acad. Sci. USA 95, 5762-5767; Will et al., 1993, J. Virol. 76, 1203-1210). EBOV GP1 (Fig. 4 lanes 5,6, and 11-12) and MBGV GP1 (Fig. 4 lanes 3-4, and 9-10) were detected in both the cell lysate and supernatant. EBOG GP2 was observed in lanes 3 and 4 at approximately 26 kDa, as expected (Volchkov 1998, supra), indicating the E/E and E/M chimeras were cleaved into GP1 and GP2 subunits. EBOV GP2 was present in the supernatant as well, although only in the context of E/E expressed protein (Fig. 4, lane 11). MBGV GP2, expected to be present at approximately 40-45 kDa (Volchkov et al., 2000, Virology 214, 421-30), was more difficult to detect is either the M/M or E/M construct. Lack of MBGV GP2 in the E/M construct was not thought to be a result of a problem with gene expression or disulfide bonding, because the MBGV GP2 was barely detectable in the M/M construct as well, and full-length GP protein was clearly produced in both instances (Fig 4, lanes 3 and 6). The MBGV GP2 subunit could either be weakly radiolabeled, or uncleaved from the GP1 subunit under these particular protein expression conditions, thus running as the full-length GP even under reducing gel electrophoresis. A band corresponding to the expected size of sGP (Wilson et al., 2000, Science 287, 1664-1666) was present in the supernatant of cultures expressing E/E and E/M (Fig. 4, lanes 11-12). This was not wholly unexpected as the transcriptional editing site of EBOV GP is upstream of the furin cleavage site, such that both constructs with EBOV GP1 subunit could potentially be edited by the polymerase during transcription and therefore produce sGP. We have

observed on multiple occasions that the VEE polymerase, in addition to the authentic viral polymerase and T7 polymerase (Volchkov et al., 1995, supra), is capable of editing the EBOV GP mRNA during transcription.

Example 2

Protection of guinea pigs with GP chimeras

Because cross protection between EBOV and MBGV GP has not been observed, the ability of the EBOV/MBGV chimeras to protect against either EBOV or MBGV infection was assessed in guinea pigs. To test protective efficacy, five groups, consisting of 12 guinea pigs each, received 2 doses of 10^6 focus forming units of replicon expressing M/M, M/E, E/E, or E/M 28 days apart. As a control, animals were also immunized with Lassa NP, an irrelevant replicon-expressed antigen. Antibody responses of animals were monitored by ELISA before each inoculation and before challenge. Half the immunized animals in each group were challenged with ca. $1000LD_{50}$ of guinea pig adapted MBGV (Musoke) and the other half with ca. $1000LD_{50}$ EBOV (Zaire) 23-26 days after the second vaccine administration (Fig. 5).

Results from the guinea pig vaccination experiment are shown in Table 3. Animals vaccinated with M/E antigen were fully protected from MBGV challenge and almost completely (5 out of 6) protected from EBOV challenge. Similarly, E/M vaccinated animals were fully protected from either EBOV or MBGV challenge. The wt E/E protected animals from EBOV challenge while M/M did not; conversely M/M protein provided protection only against MBGV challenge, as would be expected based on lack of cross-protection between the two viruses observed in previous

experiments. A single animal did survive MBGV infection after vaccination with E/E antigen, as did one animal vaccinated with the irrelevant Lassa NP protein as a negative control. These results indicate that vaccination with a chimera consisting of GP1 subunit homologous to challenge virus (E/M challenged with EBOV or M/E challenged with MBGV) fully protected animals from death. Additionally, and perhaps somewhat surprisingly, vaccination with chimeras having only the GP2 subunit homologous to virus challenge were completely (E/M challenged with MBGV) or nearly completely (M/E challenged with EBOV) protected from death.

Table 3: Protection of VRP-Chimeric GP Vaccinated Guinea Pigs from MBGV or EBOV Challenge

	MBVG Challenge		EBOV Challenge	
Antigen	S/T[a]	MDD[b]	S/T	MDD
M/M	5/5	-	0/6	10
M/E	6/6	-	5/6	8
E/E	1/6	11	6/6	-
E/M	6/6	-	66	-
LassaNP	1/6	13	0/6	10

[a]S/T, survivors/total on day 30 post infection

[b]MDD, mean day of death

Example 3

Antibody responses to vaccination and lethal virus challenge

The pre- and post-challenge antibody titers of the guinea pigs vaccinated with the chimeras are presented in Fig.6. The data indicate that before challenge, anti-MBGV specific antibodies were detected

only in the M/M, and M/E groups, and not in the E/E, E/M, or Lassa NP control vaccinated animals [MBG(pre) antigen, Fig 6 A and B]. Anti-EBOV reactive antibodies were detected in the E/E and E/M groups, but not in the M/M, M/E, or Lassa NP vaccinated animals [EBO(pre), Fig. 6 A and B]. Animals that had no detectable antibody titer to either MBGV or EBOV antigen pre-challenge showed a boost once challenged with the same virus, indicating these animals were indeed infected [MBG(post), Fig 6A; EBO(post), Fig. 6B]. There was no increase in EBOV-reactive antibodies following MBGV challenge [EBO(pre) vs EBO(post) Fig. 6A], and conversely no boost in MBGV-reactive antibodies following EBOV challenge [MBG(pre) vs MBG(post) Fig. 6B], underscoring the lack of cross-reactivity between the two viruses.Example 4

Marburg and Ebola virus post-challenge viremias

The post-challenge viremias are shown in Fig. 7. Seven days post-challenge, the Lassa-NP control animals had infectious virus in their sera, approximately 3.5 Log_{10} PFU/ml in the EBOV challenged animals (Fig. 7A) and 5 Log_{10} PFU/ml in the MBGV challenged animals (Fig.7B). Back-titration of MBGV inoculum indicated that the challenge dose was lower than expected (350pfu), which could explain the lower viremia in the MBGV-challenged LassaNP controls. Vaccination with wt GP protein derived from the challenge virus, M/M challenged with MBGV (Fig.7A) and E/E challenged with EBOV (Fig. 7B), prevented viral replication and no infectious virus was detected in these animals at day 7. Detectable viremia was also absent in MBGV-challenged animals that had been vaccinated with M/E (Fig. 7A) and in EBOV-challenged animals vaccinated with E/M (Fig. 7B). Infectious

virus was detectable in the serum of animals vaccinated with E/M and challenged with MBGV (Fig. 7A), and in animals vaccinated with M/E and challenged with EBOV (Fig. 7B). However the viremias present in those cases were at a much reduced level (~ 2-3 log decrease) compared to viremia found in the animals vaccinated with the corresponding wt GP (compare E/E with E/M vaccinated animals challenged with MBGV, Fig. 7A; M/M with M/E vaccinated animals challenged with EBOV, Fig. 7B). It is interesting to note that the LassaNP vaccinated MBGV survivor had no viremia on either day 7 or day 14. The E/E vaccinated MBGV survivor did have detectable viremia on day 7 (data not shown). One of the Lassa-NP vaccinated, MBGV challenged, animals had a delayed, but nonetheless lethal, disease course; this animal showed no viremia at day 7 but had detectable viremia (3.6 Log_{10}) at the time of death on day 14. No other animals were viremic on day 14 (data not shown). Thus, the data show that vaccination with a GP chimera containing the GP1 subunit homologous to the challenge virus prevented viral replication and induced "sterile" immunity (M/E challenged with MBGV, E/M challenged with EBOV). However, vaccination with a chimeric GP containing the homologous GP2 subunit (E/M for MBGV challenge, M/E for EBOV challenge), although preventing death in 11 out of 12 animals, did not completely prevent EBOV or MBGV replication after challenge with lethal virus dose.

Example 5

Detection of anti-GP2-reactive antibodies

Because animals vaccinated with the much smaller GP2 subunit of GP were protected from lethal virus challenge, it was of interest to know whether these

animals were mounting an antibody response to the GP2 subunit of their vaccine antigen. To begin exploring this idea, ELISAs were again performed using pre- and post-challenge sera of vaccinated animals and detergent-disrupted sucrose purified irradiated virus as antigen. Detergent disruption of antigen was carried out in 1% SDS+ 2-mercaptoethanol (2-ME), followed by 5 min at 95°C before coating ELISA plates. The data show that before challenge, E/M vaccinated animals had MBGV-reacting antibodies detectable only with detergent-disrupted virus, and not with native virus, as antigen, suggesting the presence of anti-MBGV-GP2-specific antibodies (Fig. 8A, pre-MBGV and pre-EBOV). These antibodies boosted slightly in titer following EBOV challenge (Fig. 8A, post-EBOV). Ebola-GP2 reactive antibodies were detectable, although at a lower level, in M/E vaccinated, pre-EBOV challenge animals when detergent-disrupted EBOV was used as antigen. EBOV-GP2-reactive antibodies were not detected in M/E vaccinated, pre-MBGV challenge animals (compare native and detergent disrupted EBO antigen, Fig. 8B pre-MBGV). It is unknown why anti-EBOV-GP2 reactive antibodies would be detectable in only one of the two pre-challenge groups (Fig. 8B), however it is interesting to note that a titer for the M/E vaccinated, pre-MBGV group (1.7 $\log_{10}$), similar to that seen in the M/E vaccinated pre-EBOV group, was observed when 1% Triton X-100 was used to disrupt the EBO antigen in place of SDS+2-ME (data not shown).

Western Blot analysis was also done in order to assay for the presence of GP2-specific antibodies. Sucrose purified, irradiated MBGV or EBOV antigen was separated by 4-15% SDS-PAGE, transferred to nitrocellulose, and detected with sera from vaccinated, pre-challenge animals. As shown in Fig. 9,

MBGV GP1 and GP2 were detected in sera form animals vaccinated with M/M, as would be expected as a positive control, while no MBGV-reactive antibodies were detected in sera from E/E vaccinated animals (Fig. 9, lanes 4 and 2, respectively). M/E vaccinated animals had MBGV-GP1-reactive antibodies (Fig. 9, lane 3), as expected. Anti-MBGV-GP2-specific antibodies were clearly detected in the E/M vaccinated guinea pig sera (Fig. 9, lane 1). The search for EBOV-GP2 reactive antibodies by western blot analysis using EBOV separated by SDS-PAGE as antigen was inconclusive. EBOV GP1 was detected with E/E and E/M sera, however neither the E/E nor M/E sera detected the presence of EBOV GP2 (data not shown). Thus anti-MBGV-GP2 reactive antibodies were clearly detectable by western blot analysis.

IFA was a third method used to detect the presence of either MBGV or EBOV GP2-specific antibodies in the vaccinated animals (Fig. 10). Cells mock infected or infected with VRPs expressing M/M, M/E, E/E, E/M were fixed onto teflon-etched spot slides, and stained with sera from E/M or M/E vaccinated pre-challenge animals. Convalescent E/E vaccinated, EBOV challenged and M/M vaccinated, MBGV challenged sera were used as controls. As a negative control, mock-infected cells did not react with any sera. Anti-MBGV sera detected M/M in infected cells but did not cross-react with E/E antigen; conversely anti-EBOV detected E/E protein but did not cross-react with M/M protein, as expected. Not surprisingly, anti-E/M sera detected E/E antigen, and M/E sera detected M/M antigen. Serum from M/E vaccinated animals did detected E/E antigen, suggesting the presence of anti-EBOV-GP2 specific antibodies. Serum from E/M vaccinated animals reacted, although very weakly, with cells expressing M/M

protein. Thus, using this method both anti-EBOV-GP2 and anti-MBGV-GP2 reactive antibodies can be detected in serum from guinea pigs vaccinated with the chimeric GP proteins, although anti-MBGV-GP2 reactive antibodies were detected at a much lower level compared to the anti-EBOV-GP2-reactive antibodies

In summary, wild-type MBGV and EBOV GP proteins were protective only against the virus from which they originated (type-specific immunity), as expected. However, chimeric MBGV/EBOV GP proteins were shown here to serve as dual-protective antigens in guinea pigs. In the context of a full-length (chimeric) GP, the MBGV-GP2 subunit was sufficient for protection against MBGV challenge in guinea pigs, and EBOV-GP2 protected 5/6 EBOV-challenged animals, suggesting that protective epitope(s) reside in the GP2 portion of the GP proteins. GP1 appeared to be the subunit most responsible for robust (sterile) immunity, as chimeras with the GP2 subunit homologous to challenge virus did not completely prevent viral replication in the vaccinated animal. Anti-GP2 antibody responses were subtle, but detectable with detergent-disrupted ELISA antigen, Western blot, or indirect immunofluoresence assay. Further studies to narrow down GP2-specific protective epitope(s) are underway using chimeras that have smaller segments within the GP2 subunit swapped between the EBOV and MBGV GP genes, and well as using the GP2 subunit alone as a vaccine antigen. Guinea pig T cell responses have not yet been evaluated for their role in mediating GP2-derived protection from lethal EBOV and MBGV challenge.

It remains to be determined what role the GP2-reactive antibodies play in mediating protection against lethal virus challenge for either EBOV or

MBGV. While antibody titers have not been completely predictive of outcome of disease, nonetheless they are an important component of a protective immune response. Passive transfer of anti-GP antibodies has been shown to protect guinea pigs and mice from filovirus infection (Hevey et al., 1997, supra; Hevey et al., 2002, Vaccine 20, 586-593; Wilson et al., 2000, supra). However, T-cell responses are important in the immunoregulatory response to viral infections in general, and induction of a virus-specific CTL response is presumably an important component of immune protection against filoviruses. Indeed, Wilson et al. (2001, Cell. Mol. Life. Sci. 58, 1826-1841) showed that CTL response to EBOV NP was sufficient for protection against lethal EBOV challenge in mice. Presumably a combination of both cell-mediated and humoral immune response is necessary for protection and thus it is desirable to elicit both types of responses from any filovirus vaccine. The replicon delivery system used here elicits both antibody and cell-mediated immune responses, as antigen is produced from within host cells. The results presented here indicate that protective epitope(s) reside within the GP2 subunit of the MBGV GP protein and at least partially within the GP2 subunit of the EBOV GP protein. Further investigation into the protective mechanism of the replicon-expressed chimeric GPs is therefore warranted, in order to determine the nature of the protective epitope(s), whether based on conformational GP2 protein determinants, CTL response(s) to a particular GP2 epitope, so some combination therein. Additionally these results show that a construction of a single-component bivalent vaccine protective in guinea pigs against the two most

divergent filoviruses, Ebola and Marburg, is achievable.